Toxicology and Applied Pharmacology 307 (2016) 102–107

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Terbinafine inhibits gap junctional intercellular communication

Ju Yeun Lee [a], Sei Mee Yoon [a,b], Eun Ju Choi [a], Jinu Lee [a,*]

[a] College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, 85 Songdogwahak-ro, Yeonsu-gu, Incheon 21983, Republic of Korea
[b] Department of Integrated OMICS for Biomedical Sciences, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, Republic of Korea

ARTICLE INFO

Article history:
Received 4 May 2016
Revised 14 July 2016
Accepted 30 July 2016
Available online 31 July 2016

Keywords:
Terbinafine
Naftifine
Gap junctional intercellular communication
I-YFP GJIC assay

ABSTRACT

Terbinafine is an antifungal agent that selectively inhibits fungal sterol synthesis by blocking squalene epoxidase. We evaluated the effect of terbinafine on gap junctional intercellular communication (GJIC). Fluorescence recovery after photobleaching (FRAP) and I-YFP GJIC assays revealed that terbinafine inhibits GJIC in a reversible and dose-dependent manner in FRT-Cx43 and LN215 cells. Treatment with terbinafine did not affect Cx43 phosphorylation status or intracellular Ca^{2+} concentration, well-known action mechanisms of various GJIC blockers. While a structurally related chemical, naftifine, attenuated GJIC, epigallocatechin gallate, another potent squalene epoxidase inhibitor with a different structure, did not. These results suggest that terbinafine inhibits GJIC with a so far unknown mechanism of action.

1. Introduction

Gap junctions (GJs) are channels that connect the cytosols of neighboring cells and permit the exchange of nutrients, metabolites, signaling molecules such as cyclic AMP (cAMP), Ca^{2+} and inositol trisphosphate (IP_3), and other small molecules up to 1 kDa (Alexander and Goldberg, 2003). GJs are composed of connexins. Six connexins form the channel structure, localized at plasma membrane, called connexon, which, by docking to the adjacent cell's connexon, forms a GJ. There are 21 types of human connexins named after their molecular weights (Willecke et al., 2002), and connexin 43 (Cx43) is particularly well studied.

Reflecting the functional importance of gap junctional intercellular communication (GJIC) in normal physiology and development, mutations in genes encoding connexins have been linked to several pathologies such as: peripheral neuropathy (Cx32), deafness (Cx26, Cx30, Cx31, Cx32, and Cx43), skin disorders (Cx26, Cx30, and Cx31), cataracts (Cx43, Cx46, and Cx50), and oculodentodigital dysplasia (Cx43) (Wei et al., 2004).

Depending on the context, GJIC may alleviate or aggravate tissue damage in several pathologic conditions. Toxic liver injury induced by thioacetamide and acetaminophen (Patel et al., 2012a), inflammatory response triggered by *Shigella* infection (Kasper et al., 2010), and myocardial damage caused by ischemia-reperfusion (Garcia-Dorado et al., 2004) are examples of the so-called 'kiss of death' of GJIC, which is the propagation of cell death through gap junctions. On the other hand, genetic alteration of GJIC results in enhanced neuronal vulnerability to brain ischemia (Oguro et al., 2001, Ozog et al., 2002), which substantiates the so-called 'kiss of life' of GJIC, or spatial buffering against local tissue damage via GJIC. These results suggest that GJ modulators have both therapeutic and toxicological importance.

We recently developed an iodide-yellow fluorescent protein (I-YFP) GJIC assay (Lee et al., 2015). We used the GJIC assay to screen 2320 chemicals in order to discover unreported GJ modulators. Terbinafine, one of these modulators, is an antifungal agent usually used as a cream or an ointment to treat skin mycoses. It exerts its antifungal activity by selectively inhibiting fungal squalene epoxidase (and not human squalene epoxidase) (Birnbaum, 1990). Terbinafine also increases intracellular Ca^{2+} concentration in polymorphonuclear leukocytes (Vago et al., 1994) and inhibits angiogenesis through suppression of the Rho-mediated pathway (Ho et al., 2006).

In this study, we show that terbinafine inhibits GJIC measured by fluorescence recovery after photobleaching (FRAP) assay and a recently developed I-YFP GJIC assay. We also assessed whether terbinafine affects cellular parameters associated with GJIC inhibition, such as connexin phosphorylation and changes in cytosolic Ca^{2+} concentrations.

2. Materials and methods

2.1. Cell culture

The human glioma cell lines LN215, LN215-I, LN215-YFP, and LN215-I-YFP (Lee et al., 2015) and human embryonic kidney cell line HEK 293T (ATCC), were cultured in Dulbecco's modified Eagle Medium

Abbreviations: AM, acetoxymethyl ester; CBX, carbenoxolone; Cx, connexin; EGCG, epigallocatechin gallate; FRAP, fluorescence recovery after photobleaching; GJ, gap junction; GJIC, gap junctional intercellular communication; YFP, yellow fluorescent protein.

* Corresponding author at: College of Pharmacy, Yonsei University, 85 Songdogwahak-ro, Yeonsu-gu, Incheon 21983, Republic of Korea.
E-mail addresses: whitewndus@naver.com (J.Y. Lee), sei_mee@naver.com (S.M. Yoon), yureas@naver.com (E.J. Choi), jinulee@yonsei.ac.kr (J. Lee).

http://dx.doi.org/10.1016/j.taap.2016.07.022
0041-008X/

(DMEM) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). The 1:1 mixture of DMEM and Ham's F12 media supplemented with 10% FBS was used for the FRT and FRT-Cx43 cell lines. All cultures were maintained in humidified 5% CO_2/95% air at 37 °C.

2.2. Plasmid construction

To generate the transfer plasmid pLVX-EIP-hCx43, the Cx43 coding sequence was amplified from human brain cDNA by nested PCR with the following primers: 5′- AGG AAA GTA CCA AAC AGC AG-3′ and 5′-ATA AGG CTG TTG AGT ACC ACC-3′ for external PCR; 5′- GC TCT AGA ACC ATG GGT GAC TGG AGC GC-3′ and 5′-GC TCT AGA CTA GAT CTC CAG GTC ATC AGG C-3′ for internal PCR. The amplified product was digested with *Xba*I and inserted into pLVX-EF1α-IRES-Puro (Clontech) at the XbaI site.

2.3. Lentivirus production

HEK 293T cells were plated onto six-well plates at a density of 4×10^5 cells/well and incubated for 24 h. pLVX-EIP-hCx43, psPAX2, and pMD2.G were mixed at a ratio of 4:3:1 and a total 3 μg of the mixture was transfected into HEK 293T cells in the presence of Lipofectamine2000 (Invitrogen) and the cells were further incubated for 15 h. Cells were refreshed with 2 mL of growth medium and further cultivated for 36 h. The medium containing the lentivirus was harvested and cleared by centrifugation at 3000 rpm for 3 min before storage at −80 °C.

2.4. Generation of FRT-Cx43 cell

FRT cells were grown to 30% confluence in a 24-well plate. Culture medium was changed to 1:1 mixture of fresh growth medium and conditioned medium containing the LVX-EIP-hCx43 lentivirus supplemented with 4 μg/mL polybrene (Sigma) for 15 h. The medium was then replaced with fresh medium and cells were further incubated for three days. Afterward, the cells were subcultured onto a 60-mm dish and selected with 2 μg/mL of puromycin (Sigma) for a week.

2.5. Fluorescence recovery after photobleaching (FRAP) assay

FRT-Cx43 cells were plated on a 35-mm glass-bottomed dish and grown to full confluence. Cells were loaded with 10 μM calcein-AM (Sigma) in C-solution (10 mM HEPES, pH 7.4, 140 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) for 20 min before drug treatment, as indicated. FRAP assay was performed on the laser confocal microscope LSM 710 (Zeiss). Cells in contact with more than five cells were selected and photobleached to such an extent that the ROI fluorescence was reduced below 20% of the initial fluorescence. The fluorescence images were taken 5 s before (−5 s) and right after (0 s) photobleaching, and then at an interval of 5 s for 30 s. The percentage of fluorescence recovery was calculated as follows: % fluorescence recovery $= (F_t - F_0) / (F_{-5} - F_0) \times 100$, where Ft represents the fluorescence at any time point, F_{-5}, the fluorescence recorded 5 s before photobleaching, and F_0, the fluorescence right after photobleaching. The percentage of Fluorescence recovery at 30 s represented GJIC activity.

2.6. I-YFP GJIC assay

I-YFP GJIC assay was performed as previously reported (Lee et al., 2015). The procedures are described briefly below. A mixture of LN215-I and LN215-YFP (in a ratio of 2:1) cells was plated onto 96-well plates at a density of 20,000 cells/well and grown for 24 h. After the medium was removed, cells were treated with 100 μL of C-solution containing vehicle or chemicals, as indicated. The I-YFP assay was performed well by well. The YFP fluorescence of a well was read in kinetic mode every 0.4 s for 10 s using the POLARstar microplate reader (BMG Labtech). The same volume of I-solution (10 mM HEPES, pH 7.4, 140 mM NaI, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) was injected into the well 1 s after the beginning of each experiment, with the automated injector provided with the plate reader. The percentages (%) of YFP quenching and GJIC activity were calculated as follows:

$$\%\text{YFP quenching} = \left(1 - \frac{\text{YFP fluorescence}}{\text{YFP fluorescence at 2 s}}\right) \times 100$$

$$\%\text{GJIC activity} = \frac{\%\text{YFP quenching at 10 s}}{\%\text{YFP quenching at 10 s of control group}} \times 100$$

2.7. Connexin hemichannel assay

LN215-YFP cells were grown to full confluency onto a 96-well plate. Cultures were washed twice and incubated with 100 μL of C-solution with or without Ca^{2+} for 10 min. Then, the YFP fluorescence of a well was read every 0.4 s for 30 s. For drug treatments, vehicle or drugs were diluted in the C-solution before incubation with the cells. The same volume of I-solution with or without Ca^{2+} was added with the automated injector at 1 s. Connexin hemichannel activity was determined

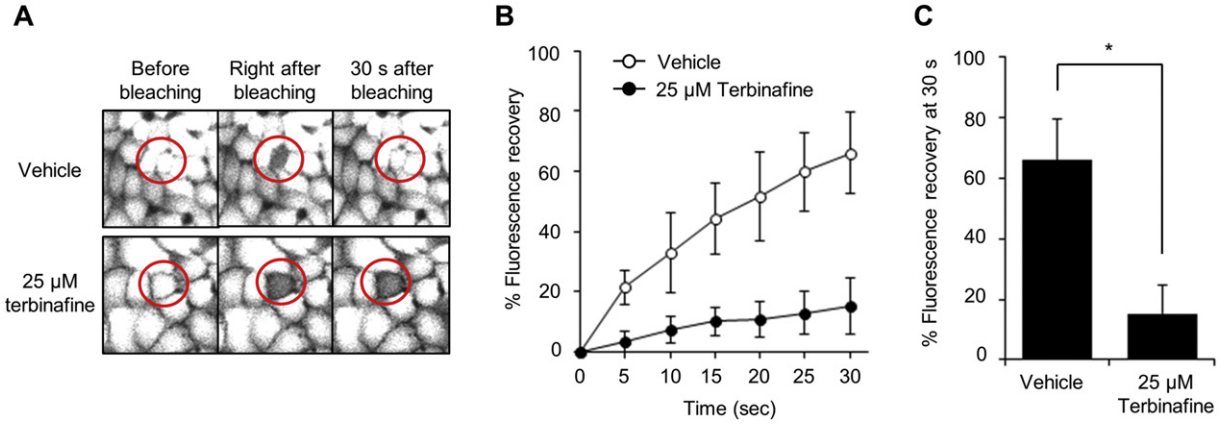

Fig. 1. Terbinafine inhibition of GJIC in FRT-Cx43 cells. FRT-Cx43 cells were loaded with calcein-AM and treated with vehicle or 25 μM terbinafine before FRAP assay. Representative images of FRAP assay were presented (A). The % of fluorescence recovery is plotted as mean ± SD (n = 6) against incubation time (B). The % of fluorescence recovery at 30 s is presented as bar graph (C). *p < 0.001 (Student's *t*-test).